International Journal of Agricultural Science and Research (IJASR)
ISSN(P): 2250-0057; ISSN(E): 2321-0087
Vol. 7, Issue 6, Dec 2017, 279-286

TRANS STELLAR
•Journal Publications •Research Consultancy

ESTABLISHMENT OF HIGH-EFFICIENCY *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF CALLUS, DERIVED FROM *SEHIMA NERVOSUM*, AN IMPORTANT RANGE GRASS SPECIES

KRISHNA KUMAR DWIVEDI[1], R. KATIYAR[2], A. RADHAKRISHNA[3]
M. K. SRIVASTAVA[4] & P. KAUSHAL [5]

[1] *Senior Scientist, Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi, (UP), India*
[2] *Senior Research Fellow, Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi, (UP), India*
[3] *Scientist, Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi, (UP), India*
[4] *Principal Scientist, Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi, (UP), India*
[5] *Principal Scientist and Joint Director Research, National Institute of Biotic stress management, Raipur (Chhattisgarh), India*

ABSTRACT

Sehima Nervosum is a natural range grass found inherently rich in precursors, for several industrially important biomolecules. Production of nutraceuticals (prebiotics xylo-oligosaccharides) from this grass is promising. It reproduces predominantly through apomixes, which provides a means of clonal propagation through seeds. The lack of sexual reproduction in S. Nervosum limits the possibilities of genetic improvement by hybridization. Development of an efficient gene delivery system would be very useful for maintenance, multiplication and genetic improvement of this grass and to enhance nutraceuticals production. A callus inducing and transformation system was developed utilizing embryogenic callus derived from seed explants of this grass on Murashige and Skoog medium with various concentrations of 2,4-D followed by infection of embryogenic calli with A. tumefaciens strain EHA105 harbouring binary vector p CAMBIA1305.1 with GUS gene. The result showed that, the number of calli expressing GUS gene first increased with inoculation time and bacterial density (OD_{600}) and then, dramatically decreased with the increment of both factors. The highest percentage (90%) of transformation efficiency was obtained in the calli, inoculated with Agrobacterium for up to 30 minutes at OD_{600} of 1.0, followed by 83% and 81% at OD_{600} of 0.6 and 1.0, respectively. Our result suggests that infection time and Agrobacterium density affects transformation efficiency. To the best of our knowledge efficient in-vitro callus induction from mature seed explants, callus transformation and transformation efficiency in Sehima is reported here for the first time.

KEYWORDS: Agrobacterium-Mediated Genetic Transformation, Sehima Nervosum Callus & GUS Gene

Received: Oct 15, 2017; **Accepted:** Nov 08, 2017; **Published:** Nov 20, 2017; **Paper Id.:** IJASRDEC201738

Original Article

INTRODUCTION

Sehima nervosum is one of the important rangeland grasses in India, It is commonly known as Saen grass in India, white grass in Australia, and has also been reported from the Central East Africa and Sudan (Samanta et al. 2012). This grass has high nutritive value and therefore, is used for grazing as well as for hay preparation for high quality fodder (Purohit et al. 1995.). It prefers hot and dry climate and survive even in limited rainfalls. As this natural grass is found inherently rich in precursors, for several industrially important biomolecules, fractionation of these precursors seems to be a promising endeavour. Production of nutraceuticals (prebiotics xylo-

oligosaccharides) from the lignocellulosic biomass of this grass is promising, as this grass does not compete with food crops, and is comparatively less expensive than conventional agricultural food-stocks (Samanta et al. 2012). However, germplasm of this grass has narrow genetic variability. Being largely apomictic in reproduction, generation of variability through hybridization approaches has been extremely limited. Utilization of biotechnological tools is one of the potential ways for introducing variability and transfer of desirable traits. The development of an efficient genetic transformation procedure for Sehima could facilitate physiological and molecular biology studies as well as the production of transgenic cultivars for higher productivity and quality.

Agrobacterium-mediated genetic transformation is the most widely used technology for plant genetic engineering mainly due to its low transgene copy number and thus, less chance to induce transgene silencing (Repellin et al. 2001; Wang et al. 2006). Agrobacterium-mediated transformation of grass species, has been reported in rice (Hiei et al. 1994), maize (Ishida et al. 1996), wheat (Cheng et al. 2003), sorghum (Zhao et al. 2000), creeping bentgrass (Yu et al. 2000), tall fescue (Wang et al. 2005), Brachypodium distachyon (Pacurar et al. 2008), buffel grass (Batra et al. 2003), italian grass (Bettany et al. 2003), perennial ryegrass (Bajaj et al. 2006), bermudagrass (Li et al. 2005) and switchgrass (Somleva et al. 2002). However, most transformation efforts of monocots still suffer from inefficiency. The present study was an attempt to develop an efficient in-vitro callus induction from mature seed explant and development of a reproducible, rapid and efficient Agrobacterium-mediated transformation in Sehima nervosum. Here we present a reproducible, rapid and efficient Agrobacterium mediated transformation using Agrobacterium strain EHA105 harbouring binary vector pCAMBIA 1305.1.

MATERIAL AND METHODS

Sehima Nervosum (Var. Bundel saen 1) seeds were used as explant materials were surface sterilized, with 70% (v/v) ethanol for 1 min, rinsed in sterile distilled water, and dipped in 0.1% (w/v) mercuric chloride solution for 10 min followed by five or six rinses with sterile distilled water. The surface sterilized seeds were inoculated on to a Murashige and Skoog (MS) basal medium (Murashige et al. 1962) containing different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mg L^{-1}) of 2,4-D (2,4-dichlorophenoxyacetic acid) (Himedia, Mumbai, India) for callus induction. The pH of MS basal medium, which contained 30 g L^{-1} sucrose (Himedia), was adjusted to 5.9 before being solidified with 0.7% (w/v) agar (Himedia) and autoclaved at 121°C for 15 min. Culture plates (90 mm Petri plates) were cultured at 26 ± 2°C in dark for four weeks for callus induction. Observations were recorded on callus induction frequency and calli were sub-cultured to the freshly prepared callus induction medium. Morphogenic changes, including callus texture, were recorded at each subculture and correlated with media components.

A. tumefaciens strains, EHA105, harboring the plasmid pCAMBIA1305.1 (Roberts et al. 1997), was used for transformation of embryogenic callus. The binary vector pCAMBIA1305.1 contains the uidA (β-glucuronidase) gene, driven by Cauliflower Mosaic Virus35S (CaMV35S) promoter and the nopaline synthase (Nos) terminator. It also contains the hygromycin phosphotransferase gene (hptII) gene as a selectable marker, driven by CaMV35S promoter and terminator. The GUS gene contained an intron in its coding region to ensure that, the observed GUS activity occurred in the plant cell and was not due to the presence of residual Agrobacterium cells. The Agrobacterium strain was inoculated in 2 mL of yeast extract broth (YEB; peptone 5 g L^{-1}; yeast extract 5 g L^{-1}; sucrose 5 g L^{-1}; magnesium sulfate 0.5 g L^{-1}; pH 7.5) supplemented with 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin. The culture was placed on a shaker at 28°C overnight, then 200 µl was then transferred to 20 mL YEB containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin and incubated at 28°C until the OD_{600} reached 0.6, 1.0 and 1.4. The culture was then centrifuged at

4000 rpm for 15 min, and the pellet was resuspended, in the same volume of liquid MS medium (Murashige et al. 1962) of pH 5.6 containing 100µm acetosyringone (Himedia), without any plant growth regulators. White and compact calli were sliced into small pieces, using sterile sharp scalpel blade and were immersed in a suspension of A. tumefaciens, harboring the plasmid pCAMBIA1305.1 (OD_{600} = 0.6, 1.0 and 1.4), for 15, 30, 45 and 60 minutes, and blotted with sterile filter paper to remove excess bacteria. Agrobacterium infected calli were then placed onto the co-cultivation medium (MS + 3.5 mg L^{-1} 2,4-D) with approx 10 pieces/plate for co-cultivation. Following 4 d of co-cultivation, the calli were washed with 300 mg L^{-1}cefotaxime and transferred to selective (the same of co-cultivation medium) supplemented with 300 mg L^{-1} cefotaxime and 30 mg L^{-1} hygromycin. Calli were sub-cultured in every 4 wk onto fresh medium of the same composition. Calli collected from transgenic and control-type was used for GUS histochemical assay as described by Jefferson (Jefferson et al. 1987). Calli were immersed in a solution of 50 mM phosphate buffer (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-gluc; Merck Biosciences, Darmstadt, Germany), 10 mM ETDA and 0.1% Triton X-100. After overnight incubation at 37°C, transient GUS gene expression was measured by counting the GUS positive calli appearing as blue zone after staining and photographed by using a Leica S6D trinocular stereozoom microscope.

RESULTS AND DISCUSSIONS

In the present study, effect of 2, 4-D on callus induction and embryogenesis from mature seed explants was observed. Surface sterilized seeds were inoculated on to a basal MS medium, containing different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mg L^{-1}) of 2, 4-D for callus induction. Mature seeds have been reported as explants source in tissue culture in range grasses such as Cenchrus (Batra et al. 2002) and Dichanthium (Bhat et al. 2001). After three weeks, callus formation was observed from seed explants. Initially, watery and loose calli were produced. Subculturing improved the growth and quality of the callus. Repeated sub culturing of these calli in MS media, containing varying concentrations of 2,4-D resulted in the formation of hard and compact embryogenic callus having somatic embryos of globular stage was observed (Fig. 1). The compact and nodular calli were used for transformation. The callus induction frequency was observed to select the best media combination, for embryogenic callus induction. The callus induction frequency were observed 40.60±1.00, 51.30±2.20, 61.40±1.60, 63.90±2.50, 70.70±2.00, 75.50±0.80, 95.60±0.30 , 64.10±2.10 and 58.10±2.23 as in 2,4-D concentration of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mg L^{-1} respectively (Fig. 2). Callus induction frequency was enhanced with the increasing concentration of 2, 4-D. The highest callus induction frequency was 95% at 3.5 mg L^{-1} 2, 4-D. Overall, 3.5 mg L^{-1} 2, 4-D showed the best callus initiation response suggesting that endogenous auxins levels might influence callus initiation. In grasses better response of 2, 4-D for callus induction has been observed (Batra et al. 2002). (Yadav et al. 2009) observed 79%, whereas Batra and Kumar (Batra et al. 2002) reported an 80% callus induction frequency in Cenchrus ciliaris. Our results are consistent with those of Batra and Kumar (Batra et al. 2002) and (Yadav et al. 2009) who also observed differences in callus induction due to various concentrations of 2,4-D.

To standardize the conditions for Sehima transformation various parameters influencing transformation frequency were examined. Especially, effect of two factors in enhancing T-DNA delivery and/or favoring callus recovery, viz. the OD_{600} of bacterial suspension and the infection time were studied. The calli were immersed in the Agrobacterium suspension at OD_{600} of 0.6, 1.0 and 1.4 for 15, 30, 45 and 60 minutes under vacuum to penetrate Agrobacterium cells in to the calli. GUS expression was histochemically assayed with X-Gluc, just after completion of co-cultivation. The difference in transformation efficiency in different studies may be attributed to the genotype of the crop species, explant type, co-

cultivation procedure followed and the Agrobacterium strains used in the genetic transformation experiments (Bhat et al. 2001; Batra et al. 2003). Calli for the stable GUS expression were sampled from the selection plates. Frequency of GUS expression was scored in the embryogenic calli. Non transgenic callus were also recorded for the expression of GUS gene activity (Fig. 3). Transformation efficiency was at OD_{600} of 0.6 of 15 min 75%, 30 min 83%, 45 min 68% and 60 min 60%, respectively. Transformation efficiency was at OD_{600} of 1.0 of 15 min 81%, 30 min 90%, 45 min 70% and 60 min 61%, respectively. Whereas, Transformation efficiency was at OD_{600} of 1.4 of 15 min 73%, 30 min 76%, 45 min 63% and 60 min 58%, respectively. The highest percentage (90%) of transformation efficiency was obtained in the calli inoculated with Agrobacterium for up to 30 minutes at OD_{600} of 1.0, followed by 83% and 81% at OD_{600} of 0.6 and 1.0, respectively (Table 1). Limited studies have been conducted in transformation in range grasses (Bhat et al. 2001; Batra et al. 2003); however there is no report on Sehima transformation. Batra and Kumar (Batra et al. 2003), reported GUS gene expression of transient callus in Cenchrus using A. tumefaciens LBA4404 harboring plasmid pCAMBIA1301. Our results are consistent but more efficient (90% transient frequency) than Batra and Kumar (Batra et al. 2003), who reported transient frequency (63%) by Agrobacterium mediated gene transfer in embryogenic calli in Cenchrus ciliaris.

CONCLUSIONS

We hereby report successfully to achieve as high as 90% efficient callus transformation in *Sehima nervosum*, for the first time. This information may be important in view of generating transgenics in this important crop. Furthermore, this exceptionally high response to callus induction and transformation in this crop showed its potential as a model crop to undertake biotechnological approaches for perennial range grass improvement.

ACKNOWLEDGMENT

This work was supported by Department of Biotechnology (DBT) in form of Rapid Grant for Young Investigators (RGYI 2012) (BT/PR6008/GBD/27/384/2012).

REFERENCES

1. *Batra S, Kumar S (2002) In vitro high frequency plant regeneration in buffel grass (Cenchrus ciliaris L.). J Plant Biol 29:191–194*
2. *Batra S, Kumar S (2003) Agrobacterium-mediated transient GUS gene expression in buffel grass (Cenchrus ciliaris L.). J Appl Genet 44:449–458*
3. *Bhat V, Dalton SJ, Kumar S, Bhat BV, Gupta MG, Morris P (2001) Particle-inflow gun-mediated genetic transformation of buffel grass (Cenchrus ciliaris L.): optimizing biological and physical parameters. J Appl Genet 42:405–412*
4. *Hanafy Ahmed A. H, Hamdy A. Moursy, Ayman Amin, Mahmoud M. Saker & Doaa H. Ali, Agrobacterium-Mediated Transformation of Super Strain B and Rio Grande Tomato Cultivars Using Gv3101 Binary Vector Harboring Ptdins-Plc2 Gene, International Journal of Bio-Technology and Research (IJBTR), Volume 5, Issue 1, January - February 2015, pp. 11-22*
5. *Bajaj S, Ran Y, Phillips J, Kularajathevan G, Pal S, Cohen D, Elborough K, Puthigae S (2006) A high throughput Agrobacterium tumefaciens-mediated transformation method for functional genomics of perennial ryegrass (Lolium perenne L.). Plant Cell Rep 25(7):651-659*
6. *Bettany A, Dalton S, Timms E, Manderyck B, Dhanoa M, Morris P (2003) Agrobacterium tumefaciens-mediated transformation of Festuca arundinacea and Lolium multiflorum. Plant cell Rep 21(5):437-444*

7. *Bhat V, Dalton SJ, Kumar S, Bhat BV, Gupta MG, Morris P (2001) Particle-inflow gun-mediated genetic transformation of buffel grass (Cenchrus ciliaris L.): optimizing biological and physical parameters. J Appl Genet 42:405–412*
8. *Cheng M, Hu T, Layton J, Liu CN, Fry JE (2003) Desiccation of plant tissue post-agrobacterium infection enhances T-DNA delivery and increase stable transformation efficiency in wheat. In Vitro Cell Dev Biol Plant 39:595-604*
9. *Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequences-analysis of the boundaries of the T-DNA. Plant J 6:271-82*
10. *Ishida Y, Saito S, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (Zea mays L.) mediated by Agrobacterium tumefaciens. Nat Biotechnol 14:745-50*
11. *Jefferson RA, Kavanagh, TA, Bevan, MW (1987) GUS Fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901-3907*
12. *Li L, Fei S, Qu R (2005) Agrobacterium-mediated transformation of common bermudagrass (Cynodon dactylon). Plant Cell Tiss Organ Cult 83:223–229*
13. *Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bioassay with Tobacco Tissue Culture. Plant Physiology 15:473-97*
14. *Purohit SD, Kukda G (1995) Embryogenesis and plant regeneration from tissue culture of Sehima nervousum (Rottl.) Stapf. Bionature 15:27-30*
15. *Pacurar DI, Hans TC, Kristian K, Lenk NI (2008) A high-throughput of Agrobacterium-mediated transform system for the grass model species Brachypodium distachyon L. Transgenic Res 17:965-75*
16. *Repellin A, Baga M, Jauhar PP, Chibbar RN (2001) Genetic enrichment of cereal crops via alien gene transfer: new challenges. Plant Cell Tissue Organ Cult 64:159-83*
17. *Roberts CS, Rajagopal S, Smith L, Nghyen T, Yang W, Nugroho S, Ravi RS, Cao ML, Chandra KV, Pattell V, Harcourt R, Dransfield L, Desamero N, Slamst I, Keese P, Kilian A, Jefferson RA (1997) A comprehensive set of modular vectors for advanced manipulation and efficient transformation of plants. Rockefellar Found. Meet. Intern. Program on Rice Biotechnology, 15-19 Sep. Malacca, Malaysia*
18. *Samanta AK, Natasha J, Kolte AP, Senani S, Sridhar M, Suresh KP, Sampath KT (2012) Enzymatic production of xylooligosaccharides from alkali solubilized xylan of natural grass (Sehima nervosum). Bioresource Technology 17(112):199–205*
19. *Somleva, MN, Tomaszewski Z, Conger BV (2002) Agrobacterium-mediated genetic transformation of switchgrass. Crop Sci 42:2080–2087*
20. *Wang ZY, Ge Y (2006) Recent advances in genetic transformation of forage and turf grasses. In Vitro Cell Dev Biol Plant 42:1-18*
21. *Yadav CB, Jha P, Mahalakshmi C, Anjaiah V, Bhat V (2009) Somatic embryogenesis and regeneration of Cenchrus ciliaris genotypes from immature inflorescence explants. Biol Plantarum 53: 603-609*
22. *Yu TT, Skinner DZ, Liang GH, Trick HN, Huang B (2000) Agrobacterium-mediated transformation of creeping bentgrass using GFP as a reporter gene. Hereditas 133: 229-33*
23. *Zhao ZY, Cai TS, Tagliani L, Miller M, Wang N, Pang H (2000) Agrobacterium-mediated sorghum transformation. Plant Mol Biol 44:789-98*

APPENDICES

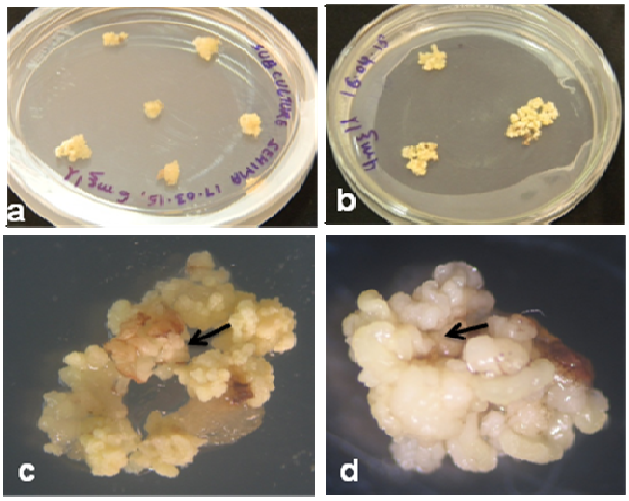

Figure 1: In-vitro callus induction and somatic Embryogenesis from Seed Explants of Sehima Nervosum: (a) callus Induction on Embryogenic Callus Induction Medium (ECIM) Containing 3.5 mg/l 2,4-D after 3 wk; (b) Development of Somatic Embryos on embryogenic Callus Induction Medium Containing 3.5 mg/l 2,4-D after wk (c) Close up of Embryogenic Callus With Proembryogenic Masses (PEM) on Embryogenic Callus Induction Medium Containing 3.5 mg/l 2,4-D after 4 wk (arrow indicates PEM); (d) Somatic Embryos (SEs; globular, Heart, Torpedo, and Cotyledonary stage) on Embryogenic Callus Induction Medium (Arrow Indicates)

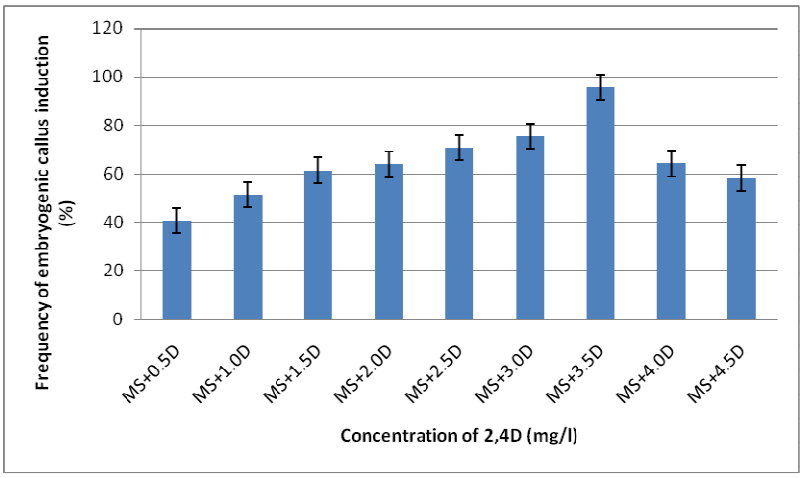

Figure.2: **Influence of Different Concentrations of 2, 4-D on Embryogenic Callus Induction from Mature Seeds of S. Nervosum**

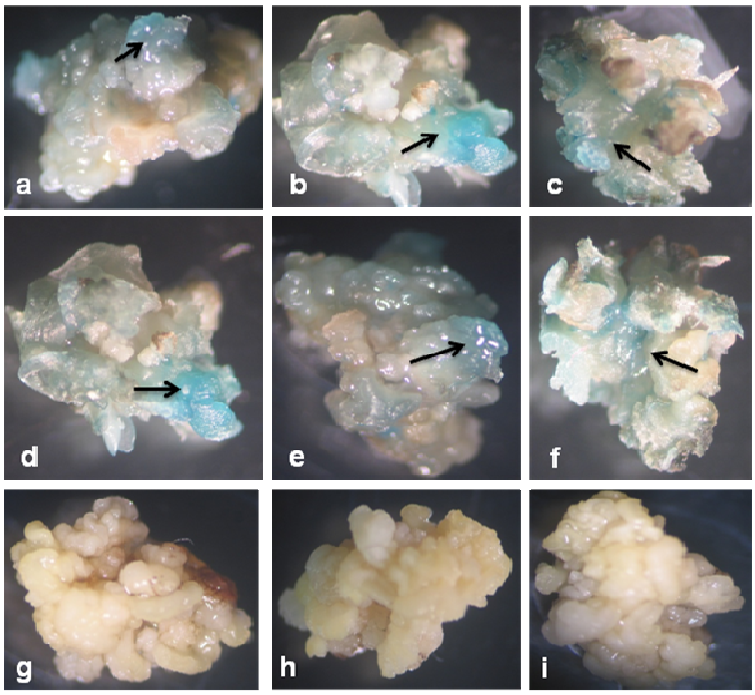

Figure 3: Agrobacterium-Mediated Transient GUS Gene Expression in SEHIMA Nervosum: (a, b, c, d, e and f) Transient GUS Gene Expression in Embryogenic Calli (indigenous blue color) after Co-Cultivation With A. Tumefaciens Strain EHA105/pCAMBIA-1305.1 (arrow indicates); (g, h and i) No GUS Gene Expression in Control.

Table1: Effect of Different Infection Time and Bacterial Concentration on Sehima Calli Transformation Efficiency

Infection time (min.)	OD_{600}								
	0.6			1.0			1.4		
	No. of Calli Infected	No. of GUS Expressing Calli	T%*	No. of calli Infected	No. of GUS Expressing Calli	T%*	No. of Calli Infected	No. of GUS Expressing Calli	T%*
15	60	45	75.00	60	49	81.66	60	44	73.00
30	60	50	83.33	60	54	90.00	60	46	76.66
45	60	41	68.33	60	42	70.00	60	38	63.33
60	60	36	60.00	60	37	61.00	60	35	58.33

*T%: Transformation efficiency %